PATENT
2173-0106P

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of Before the Board of Appeals

Yuji HATADA et al. Appeal No.:

Appl. No.: 08/952,741 Group: 1652

Filed: November 25, 1997 Examiner: E. Slobodyansky

Conf.: 3031

For: GENE ENCODING ALKALINE LIQUIFYING ALPHA-AMYLASE

REPLY BRIEF TRANSMITTAL FORM

Assistant Commissioner for Patents
Washington, DC 20231

October 15, 2002
(Tuesday following Holiday)

Sir:

Transmitted herewith is a Reply Brief (in triplicate) on behalf of the appellants in connection with the above-identified application.

☐ The enclosed document is being transmitted via the Certificate of Mailing provisions of 37 C.F.R. § 1.8.

The Examiner's Answer was mailed on August 13, 2002.

☐ An extension of time under 37 C.F.R. § 1.136(b) to was requested on and was approved on .

☐ Please charge Deposit Account No. 02-2448 in the amount of $0.00. A triplicate copy of this sheet is attached.

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Respectfully submitted,

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(Rev. 01/02/02)

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:	Yuji HATADA et al.	Conf.:	3031
Appl. No.:	08/952,741	Group:	1652
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#35 (9.J) 10/28/02

REPLY BRIEF

Assistant Commissioner for Patents
Washington, DC 20231

October 15, 2002
(Tuesday following Holiday)

Sir:

In response to the Examiner's Answer of August 13, 2002, the following Reply Brief is submitted in connection with the above-identified application.

Withdrawn Rejections and Pending Rejections

The Examiner has acknowledged in the Examiner's Answer of August 13, 2002 that the 35 USC §112, first paragraph, new matter rejection and the 35 USC §103(a) rejection over Ara et al. in view of Tsukamoto et al. or Yuuki et al. have been withdrawn. Accordingly, only two rejections remain: 1) the 35 USC §112, first paragraph, written description rejection and 2) the 35 USC §112, first paragraph, enablement rejection. This

Reply Brief addresses new points of argument made by the Examiner in the Examiner's answer on these issues.

Remarks to rebut Examiner's assertions

Regarding written description, at page 6, lines 7-10 of the Examiner's answer the Examiner states:

> *Such variant and a DNA encoding thereof encompass a great number of molecules, both naturally occurring and synthetic, encoding amino acid sequences some of which may not have any structural homology with SEQ ID NO: 2.*

Appellants do not agree with the Examiner's assertion that the variants of the instant invention may not have any structural homology with SEQ ID NO: 2. This may be the case if nothing were known about amylases. It appears to the Appellants that the Examiner is examining the claim in a vacuum without taking into consideration what is known in the art about amylases. At the last 4 lines of page 15 of the Appeal Brief submitted May 13, 2002, Appellants note that amylases are known to have four highly conserved regions, designated regions I-IV wherein structural changes are unlikely to occur without a change in the enzymological properties (see claim 1) of the polypeptide.[1]

[1] As proof that these conserved regions were known prior to the filing of the instant application, Appellants submit the following reference demonstrating these conserved regions: Nakajima , R. et al. Appl. Microbial. Biotechnol., 23 pp. 355-360, (1986). In particular, these conserved regions are as follows (residue numbers refer to SEQ ID NO: 2). Conserved Region I is from Asp133-His 138 and are made up of the following amino acids: AspValValMetAsnHis. Conserved Region II is from Gly263-His271 and is made up of the following amino acids:

Thus, the skilled artisan is unlikely to change these parts of the protein and furthermore, any changes as are made in these regions will likely be conserved amino acid substitutions. Accordingly, Appellants assert that the variants of the instant invention will have structural homology with SEQ ID NO: 2.

Regarding written description, at page 6, line 17 (last paragraph) to the first line of page 7, the Examiner states:

> *The specification . . . fails to provide any structure: function correlation present in all members of the claimed genus nor they are [SIC, are they] known in the art.*

The Examiner's implication that there is no structure:function correlation defining the claimed genus is incorrect. As was pointed out above, Appellants assert that because amylases are known to have four highly conserved regions, designated regions I-IV, wherein structural changes are unlikely to occur, it is not accurate to state that no structure:function correlation is known in the art.

At page 8, lines 3-6, the Examiner states:

> *While the combination of the properties described in claim 21 is imparted by the specific structure and therefore, reflects a certain degree of "structural limitations", those are properties of an enzyme not a DNA. However, an adequate written description of DNA requires the definition of the specific properties of a DNA itself.*

GlyPheArgIleAspAlaValLysHis. Conserved Region III is from Glu297-Lys300 and is made up of the following amino acids: GluPheTrpLys. Conserved Region IV is from Phe359-Asp364 and is made up of the following amino acids: PheValAspAsnHisAsp.

Again, Appellants assert that this statement makes it appear as if the Examiner is examining in a vacuum. If one knows what the primary sequence is of an enzyme that is coded by a DNA, one necessarily knows what that DNA sequence is (with some variation accounting for several different codons that can code for a given amino acid and some variation due to the "wobble" position). Appellants assert that claim 21 is a finite set and thus, Appellants further assert that every single possible DNA that fits into this set can be identified. In other words, by providing a description of the enzyme, Appellants have described the DNA that falls within the scope of this claim. The fact that this is indeed a finite set was pointed out in the Appeal Brief at page 22, lines 1-11.

Regarding enablement, at page 12, line 12 to page 13, line 3, the Examiner states:

> *The specification provides guidance and examples for obtaining DNAs encoding an a-amylase having an amino acid sequence of SEQ ID NO: 2 from Bacillus sp. KSM-AP1378 and its N-terminal deletion mutant. While molecular biological techniques and genetic manipulation to make and use the claimed nucleic acid sequences are known in the prior art and the skill of the artisan are well developed, knowledge regarding the amino acid residues which are important to the enzymatic activity and folding of the Δ-amylase, the amino acid residues which can be inserted into or deleted from the amino acid sequence of SEQ ID NO: 2 without affecting the requisite specific enzymatic activity, amino acid homology among Δ-amylases with said specific enzymatic activity from various biological sources, and the nucleic*

acid sequences encoding said Δ-amylases from various biological sources is lacking.

The Examiner in this paragraph fails to acknowledge that in addition to the guidance indicated in this paragraph, Appellants have also provided guidance in using the primers on the template DNA from Bacillus species. In the specification, please see the last paragraph on page 4, page 6, lines 6-10 and page 7, lines 1-12. Because these DNA primers were designed based on the highly conserved regions from the enzymes, the procedure elucidated in these paragraphs is a general procedure, which allows one to isolate other nucleic acids encoding variant proteins. The skilled practitioner can express these variants as described in the Examples and confirm that they retain the enzymological properties compared to the protein of SEQ ID NO: 2 (claim 1) by performing the assays described in Example 7. Accordingly, Appellants assert that following these procedures would allow one of skill in the art to make and use the instantly claimed invention without undue experimentation.

At page 16, lines 1-4 of the Examiner's Answer, the Examiner states:

Appellants argue that they "have provided how one would screen recombinant microorganisms to identify those expressing an enzyme according to claim 3 [SIC "] (page 19 1st full paragraph). This is not agreed with as the point of the rejection is description not the enablement.

Appellants assert that the point of the paragraph at page 19, first full paragraph of the Appeal Brief is to show that the specification describes the recited enzymological properties in claim 3. One of those properties is that the protein having the amino acid sequence of SEQ ID NO: 2 or a modified version of SEQ ID NO: 2 has the ability to cleave a glucosidic linkage. Appellants have thus described the claim term "enzymological properties". It also happens that Appellants have described how variant proteins can be identified as within the scope of claim 1 and especially claim 3.

Accordingly, although at first glance one might surmise that the sentence quoted by the Examiner (the sentence that is cited in the above quote) refers to an enablement issue, it also refers to a written description issue because it shows that Appellants have described a claim term as well as how proteins of the invention can be identified.

At page 16, lines 15-19 of the Examiner's Answer, the Examiner states:

> *Appellants refer to the conserved regions in amylases as structural limitations provided by the art (page 22, 2nd paragraph). It is apparent that these conserved regions are not responsible for the specific properties recited in the claim as changes in the structure as little as about 15% lead to drastic changes in properties, supra.*

The above statement illustrates precisely the point that Appellants assert. By changing amino acids in the conserved regions, one loses function. Thus, one of skill in the art would not change these conserved regions. Accordingly, Appellants assert that these conserved regions provide additional structural elements. When one considers these structural elements, Appellants assert that they have sufficient written description to demonstrate that the invention was in full possession of the Appellants at the time of filing the instant specification.

At page 17, lines 1-9 of the Examiner's Answer, the Examiner states:

> *Appellants further argue that claims 22-24 are adequately described because the claims "have both structural and functional language" (page 23). Appellants assert that "there is no ambiguity in this structural element. No variants in this structural element are claimed" (page 24, 1st full paragraph). The recited structural element of the genus encompassed by claims 22-24 does not constitute a substantial porting of the genus as the remainder of the structure of a polypeptide with a-amylase activity is completely undefined. Fragments consisting of 20-26 nucleotide [SIC nucleotides] of SEQ ID NO: 1 are highly unlikely to encode a-amylase activity and the specification does not define the remaining structural features necessary for members of the genus to be selected.*

The Examiner fails to acknowledge that these fragments are incorporated into longer molecules during PCR (please see page 7, lines 1-12 of the specification). Accordingly, Appellants

assert that these claims have a structural feature (i.e. the structure of the primer) and a functional feature (i.e., encoding an enzyme exhibiting Δ-amylase activity at a pH optimum of 8-9), which readily allows quick determination of whether a DNA falls into the claimed genus or not. As such, the invention was fully in possession of the Appellants at the time of filing the application.

At page 18, lines 13-14 of the Examiner's Answer, the Examiner states:

> *As discussed above, there is no guidance as to what residues can be modified without affecting the properties.*

Appellants assert that while predictability is a factor that is to be considered when examining enablement, the requirement is not one of *a priori* structure prediction. Rather the question is whether given the teachings of the specification and the prior art, is it predictable that an embodiment of the invention can be made? In *In re Wands*, 8 USPQ 2d 1400 (Fed. Cir. 1988) the Court of Appeals for the Federal Circuit found that the claims directed to antibodies were enabled even though the Court acknowledged that the antibody field was a highly unpredictable field. The Court found that the claims were enabled because the applicants for patent had demonstrated a method of screening hybridomas for active antibodies. The invention was deemed

enabled despite a success rate of a mere 2.8% and complete failure of two experiments. Similarly, the instant application provides a method for screening mutants by looking for glucosidic bond cleavage activity. Appellants have in fact shown a method of how to make mutant proteins within the scope of the claims without undue experimentation.

At page 19, lines 1-4 of the Examiner's Answer, the Examiner states:

> *. . . the facts in the instant application and in the Wands case are different. Wands et al. provides [SIC provide] the guidance for immunizing the animal with the compound of a known structure (HbsAg) and then further screening cells for negative hybridomas.*

Appellants assert that the facts are not as different as the Examiner asserts. The instant case is similar to *Wands* in that a compound of known structure, (i.e., a primer) is used with the chromosomal DNA of an alkaline liquefying Δ-amylase-producing microorganism (as a template) and then screening is done to isolate the enzyme with the desired properties. (see page 7, lines 1-12 and Example 7 on page 17 of the specification). Screening for clones that contain a cDNA of interest by an enzymological property (*i.e.*, ability to cleave glucosidic linkages) is well known in the art. See, for example, Joseph Sambrook, Edward F. Fritsch, & Thomas Maniatis, Molecular

Cloning A Laboratory Manual, pp. 8.46-8.63, (2nd ed. Cold Spring Harbor Laboratory Press 1989, attached as Exhibit A). Accordingly, Appellants contend that in light of what one of skill in the art knows and the teachings of the instant written description, the instant application shows how to make and use the invention without undue experimentation, and thus is fully enabled for the full claimed scope of the invention.

Conclusion

For the reasons advanced above as well as for the reasons that were enumerated in the Appeal Brief filed May 13, 2002, Appellants assert that the claims, as currently pending, have adequate description under the provisions of 35 USC §112, first paragraph. Further, Appellants assert that the appealed claims are fully enabled for the full scope of the invention. Appellants assert that the Examiner's rejections are inapposite. Reversal of the Examiner's rejections under 35 USC §112, written description and enablement by the Honorable Board is warranted and respectfully requested.

With the above remarks, it is believed that the claims, as they now stand, define patentable subject matter and urge

reversal of the Examiner's rejections and issue of said claims in a U.S. Patent.

If any questions remain regarding the above matters, please contact Appellants' representative, Mark J. Nuell (Reg. No. 36,623), in the Washington metropolitan area at the phone number listed below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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IDENTIFICATION OF cDNA CLONES OF INTEREST

Methods of Screening

There are three methods to screen cDNA libraries for clones of interest:

- Nucleic acid hybridization
- Immunological detection of specific antigens
- Sib selection either by hybrid selection and translation of mRNA or by production of biologically active molecules

Most cloning projects today are aimed at isolating cDNAs corresponding to rare mRNAs and therefore require screening of large numbers of recombinant clones. This can be carried out effectively with only two types of reagents: antibodies and nucleic acid probes. In those rare instances when both types of reagents are available, nucleic acid probes are preferred because they can be used under a variety of different stringencies that minimize the chance of undesirable cross-reactions. Furthermore, nucleic acid probes will detect all clones that contain cDNA sequences, whereas antibodies will react only with a subset of these clones (in some cases one in six at best) in which the cDNA has been inserted into the vector in the correct reading frame and orientation. cDNA libraries that are to be screened by antibodies therefore need to be larger (by a factor of at least 6) than those that are to be screened by nucleic acid probes. Consequently, when using antibody probes to search for a cDNA clone corresponding to a mammalian mRNA present at the level of 1 molecule/cell or less, it is desirable to construct cDNA expression libraries that contain in excess of 10^7 members. This is not easy, especially when the amounts of mRNA are limited. Furthermore, screening a library of this size is expensive and laborious, and it becomes worthwhile to explore methods to enrich the mRNA (or cDNA derived from it) for the sequences of interest (see pages 8.6–8.10).

NUCLEIC ACID HYBRIDIZATION

This is the most commonly used and reliable method of screening cDNA libraries for clones of interest. None of the other methods displays such an abundance of attractive features. Screening by nucleic acid hybridization allows extremely large numbers of clones to be analyzed simultaneously and rapidly, does not require that the cDNA clones be full-length, and does not require that an antigenically or biologically active product be synthesized in the host cell. Furthermore, as a result of more than 20 years of work, the theoretical basis of nucleic acid hybridization is well-understood. This has led to the development of a large number of different techniques that can accommodate nucleic acid probes of very different lengths and specificities. Details of the methods for the preparation and use of these probes are presented in Chapters 10 and 11.

Homologous probes

Homologous probes contain at least part of the exact nucleic acid sequence of the desired cDNA clone. They are used in a variety of circumstances, for

example, when a partial clone of an existing cDNA is used to isolate a full-length clone from a cDNA library. Usually, a fragment derived from one end or the other of the existing clone is isolated, radiolabeled in vitro, and used to probe a library. Hybridization with homologous probes is always carried out under stringent conditions.

Partially homologous probes

Partially homologous probes are used to detect cDNA clones that are related, but not identical, to the probe sequences. If neither antibody nor nucleic acid probes are available, a number of alternative strategies can be considered. For example, if the same gene has already been cloned from another species or if a related gene has been cloned from the same species, it would be worthwhile carrying out a series of trial experiments to determine whether there is sufficient conservation of nucleic acid sequence to allow the screening of a cDNA library by hybridization. This is most easily accomplished by performing a series of Southern and northern hybridizations at different stringencies. For example, a large batch (50 μg) of genomic DNA is cleaved with a restriction enzyme that cleaves the probe sequence at one or two well-separated sites. It is a good idea to digest an equal amount of genomic DNA of the original species for use as a positive control. Aliquots (5–10 μg) of the digests are then applied to adjacent slots of a 0.8% agarose gel, electrophoresis is carried out, and the fragments are then transferred to a nitrocellulose filter as described in Chapter 9, pages 9.34–9.41. The filter is cut into strips, each of which is hybridized under different conditions to identical amounts of radioactive probe. For aqueous hybridization, the ionic strength of the solution is kept constant (usually 1 M Na^+) while the temperature of annealing is progressively lowered (from 68°C to 42°C). The strips are then washed extensively at the temperature of hybridization with a solution containing 2 × SSC, 0.5% SDS. When hybridization is carried out in solvents containing formamide, the temperature and ionic strength are usually kept constant (42°C and 6 × SSC [or 6 × SSPE], respectively) while the amount of formamide in the annealing buffer is progressively lowered from 50% to 0%. The strips are then washed extensively at 50°C in 6 × SSC, 0.5% SDS. A similar series of hybridizations can be carried out with mRNA preparations that have been fractionated by electrophoresis and transferred to a solid support. In both cases, the aim is to establish conditions that will allow the previously cloned gene to be used as a probe for the cDNA of interest, without undue interference from background hybridization.

Total cDNA probes

Total cDNA probes are prepared by uniform incorporation of radiolabeled nucleotides with reverse transcriptase or end-labeling of total or fractionated poly(A)$^+$ mRNA. They can be used to screen libraries of cDNA for specific clones *if* the cDNA clones of interest correspond to mRNA species present in the initial population at a frequency of at least 1 in 200 (see Gergen et al. 1979; Dworkin and Dawid 1980). It is not possible to detect cDNA clones homologous to species that are represented rarely in the mRNA preparation.

Subtracted cDNA probes

Subtracted cDNA probes are often used to probe cDNA libraries for clones that correspond to mRNAs that are differentially regulated. A cDNA probe prepared from one type of mRNA is depleted of sequences that are present in a second type of mRNA by subtractive hybridization (Timberlake 1980; Zimmerman et al. 1980). Typically, the cDNA is hybridized two or three times in succession to a 20-fold excess of the second mRNA, and the cDNA:mRNA hybrids are removed by chromatography on hydroxyapatite. The unhybridized cDNA is then annealed to a 100-fold excess of the mRNA preparation from which it was originally synthesized, and the resulting cDNA:mRNA hybrids are this time recovered by chromatography on hydroxyapatite. After the mRNA is removed by alkaline hydrolysis, the cDNA, which is highly enriched for sequences specific to the original mRNA, is used to probe a cDNA library for clones homologous to these sequences.

Subtracted cDNA probes are particularly valuable when there are very few differences between the two starting mRNA preparations, i.e., when most species of mRNA are represented equally in the two preparations and a small proportion (<2%) of the mRNAs are not present at all in one preparation. cDNAs that have been cloned using subtracted cDNA probes include the murine J immunoglobulin chain (Mather et al. 1981) and the murine T-cell receptor (Hedrick et al. 1984).

A slightly different approach is used when two preparations of mRNA share sequences that are present at different concentrations. Examples of such sib pairs might be mRNAs extracted from control cells and cells that have been exposed to heat shock, drugs, or hormones. cDNAs corresponding to mRNAs whose expression is altered by such treatments can often be detected by *differential hybridization*. ^{32}P-labeled first-strand cDNAs are synthesized in vitro using both mRNAs as templates. Most of the cDNA sequences correspond to mRNAs whose concentrations are not appreciably changed by the treatment to which the cells were exposed. However, a minority of the cDNAs will be copied from mRNAs whose concentrations are significantly increased or decreased. The two cDNA probes are then used to screen replicas of a cDNA library constructed from mRNA extracted from control cells (when searching for mRNAs that are repressed) or treated cells (when searching for mRNAs that are induced). The clones that hybridize preferentially to one of the cDNA probes are chosen for further analysis. Among the many examples of genes cloned in this way are the galactose-inducible genes of yeast (St. John and Davis 1979), human fibroblast interferon (Taniguchi et al. 1980), the glucose-regulated proteins of mammalian cells (Lee et al. 1981), growth-related proteins (Foster et al. 1982; Cochran et al. 1983; Linzer and Nathans 1983), differentiation-specific proteins (Spiegelman et al. 1983), and a variety of heat-shock proteins and stress proteins (see, e.g., Mason et al. 1986). The procedure has also been used to identify cDNA clones of developmentally regulated mRNAs from organisms of many different species including *Xenopus* (Williams and Lloyd 1979; Weeks et al. 1985), *Dictyostelium* (Rowekamp and Firtel 1980; Mehdy et al. 1983), sea urchins (Lasky et al. 1980), and mice (Gorman et al. 1985).

Synthetic oligonucleotide probes

Synthetic oligonucleotide probes are tracts of dNTPs of defined sequence that have been synthesized in vitro. The sequence of these probes is deduced, using the genetic code, from short regions of the known amino acid sequence of the protein of interest. Because of the degeneracy of the genetic code, it is very unlikely that a given sequence of amino acids will be specified by a predictable single oligonucleotide of defined sequence. Instead, in the vast majority of cases, the same sequence of amino acids can be specified by many different oligonucleotides. There is no way to know with certainty which of these oligonucleotides is actually used in the gene of interest. Three solutions have been found to this problem:

1. A family of oligonucleotides can be synthesized containing all possible sequences that can code for a given sequence of amino acids. The number of members in this family depends on the degree of degeneracy of the codons for the particular amino acids. However, since all possible oligonucleotide sequences are represented, at least one of the members will match perfectly with the cDNA clone of interest. To keep the size of each family within manageable proportions, short oligonucleotides (14–17 nucleotides) are generally used—the minimum size that is practical for hybridization. Often, more than one family of oligonucleotides is synthesized based on separate sequences of amino acids.

2. A longer (40–60-base) oligonucleotide of unique sequence can be synthesized using the most commonly used codon for each amino acid. (Avoid using the dinucleotide CpG, since it is underrepresented in most eukaryotic DNAs.) Almost certainly, this oligonucleotide will not match exactly the sequence in the cDNA, but it will fit well enough to be detected by hybridization under nonstringent conditions.

3. An oligonucleotide can be synthesized that contains a base such as inosine at positions of high potential degeneracy. Inosine can pair with all four conventional bases without seriously compromising the stability of the resulting hybrid. It is therefore possible to generate families of longer oligonucleotides that are reduced in number and yet are capable of hybridizing to virtually all cDNA clones that are likely to code for the protein of interest.

Finally, if the protein sequence available is from the amino terminus of the protein, the cDNA library that is to be screened must be of high quality to ensure that most of the 5′ terminus of the mRNA is represented. For a detailed discussion of synthetic oligonucleotide probes, see Chapter 11.

IMMUNOLOGICAL DETECTION OF SPECIFIC ANTIGENS

cDNA libraries constructed in expression vectors such as λgt11, λgt18–23, λZAP, and λORF8 can be screened with antibody directed against the protein of interest (see Chapter 12 for experimental details). Nitrocellulose filters imprinted with the detritus of bacterial lysis are soaked in a solution

containing the antibody. After washing, the filter is incubated with *Staphylococcus aureus* protein A or with a second antibody directed against the species-specific epitopes of the first antibody. In the original descriptions of the method, the secondary ligand was radiolabeled with ^{125}I. Today, the secondary ligand is covalently linked to an enzyme whose activity can be detected histochemically (e.g., alkaline phosphatase).

The key to success with this method lies in the quality of the antibody. It is essential that the antibody efficiently recognize the denatured protein (i.e., it should produce strong signals on western blots). Screening is made easier and more sensitive if, in addition, the antibody is derived from a polyclonal antiserum of high titer. Because such antisera normally react with many different epitopes, the chances of detecting a cDNA clone that expresses a fragment of the protein of interest are increased. Polyclonal antisera often contain cross-reacting antibodies that recognize nonrecombinant components of the bacterial lysate; these must be removed before screening is undertaken (see Chapter 12).

The background of nonspecific binding is much lower when a monoclonal antibody is used as a probe. However, the number of recombinants that can be detected is also reduced, because each individual monoclonal antibody can react with only a single epitope. The ideal immunological probe might therefore consist of a cocktail of several different monoclonal antibodies, each of which reacts strongly with denatured protein.

SIB SELECTION OF cDNA CLONES

Two methods of screening are based on the concept of dividing a large cDNA library into a manageable number of pools, each consisting of between 10 and 100 clones. These pools are then tested for the sequence of interest. The lower complexity of each pool allows methods that are relatively insensitive to be applied to a complete cDNA library. After a pool is identified that scores positively, it is subdivided into successively smaller and smaller pools, each of which is retested until the cDNA clone of interest is isolated. The process of sib selection and analysis can sometimes be performed more rapidly if, during subdivision of the initial pool, the individual clones are assigned to their subpools in a matrix format (Wong et al. 1985). This expands the number of subpools by a factor of 2 or 3, but it often leads directly to the identification of the clone of interest.

Hybrid selection

In this method, cDNA clones carrying sequences complementary to specific mRNAs are denatured, immobilized on a solid matrix, and hybridized to preparations of mRNA. The mRNA:cDNA hybrids are then heated to release the mRNA, which is then translated in a cell-free protein-synthesizing system or in *Xenopus* oocytes. The translation products are identified by immunoprecipitation and/or SDS-polyacrylamide gel electrophoresis (see Chapter 18).

Today, this procedure is used mainly to confirm the identification of cDNA clones isolated by other means. However, before the development of methods to screen cDNA libraries with immunological or oligonucleotide probes, it was

also used occasionally for the primary isolation of cDNA clones. For example, Parnes et al. (1981), using DNA from pools that contained as many as 100 individual cDNAs cloned in plasmids, were able to detect specific translation of an mRNA that represented approximately 0.03% of the total cellular mRNA. This entire procedure is labor-intensive and is worth attempting only as a last resort with mRNAs that can be greatly enriched by size selection and for which no other probes are available.

Production of biologically active molecules

A few groups have used sib selection and analysis to screen cDNA libraries for the production of biologically active protein molecules in cells. For example, in vitro assays have been used to identify cDNA clones that express the human lymphokines colony-stimulating factor and interleukin-3 in cultures of mammalian cells (Wong et al. 1985; Yang et al. 1986). This approach is usually undertaken when no other methods of screening are available and when the protein product is small enough to give reasonable assurance that the cDNA library will contain full-length clones.

Methods to Validate Clones of cDNA

cDNA libraries are usually plated at high density for screening with antibody or nucleic acid probes, and any clones that react positively in the first round require several additional cycles of plating and screening before they can be considered pure. However, the ability to react consistently with a particular probe, although an encouraging and necessary property, is not sufficient to prove that a given cDNA clone is derived from the mRNA of interest. The only absolute proof of identity is to show that the cDNA clone contains an open reading frame that codes for the entire amino acid sequence of the protein. Since this is clearly impractical for most proteins of current interest, other, less elemental tests must be used. In decreasing order of rigor, these include:

- Expression from the full-length cDNA in prokaryotic or eukaryotic cells of a protein that displays the correct biological or enzymatic activity.
- Correspondence between portions of the nucleotide sequence of the cDNA and the amino acid sequences of peptides derived from the purified protein.
- Correspondence between the peptide maps of the polypeptide synthesized in vitro by transcripts of the cDNA clone and peptide maps of the authentic protein.
- Immunoprecipitation of the polypeptide synthesized in vitro or in vivo from transcripts of the cDNA clone by antibodies raised against the protein of interest. The stringency of this test increases when it is carried out with a series of monoclonal antibodies that recognize different epitopes on the protein.
- Immunoprecipitation of the authentic protein with antibodies raised against synthetic peptides whose sequences are determined by the nucleic acid sequence of the cloned cDNA.

It is essential that the tests used to validate a cDNA clone do not use the same reagents that were used to identify the clone in the first place. If, for example, a clone is selected by screening a library with antibody, it cannot be validated in tests that use the same antibody (such as immunoprecipitation of polypeptides synthesized in vitro from transcripts of the clone). In addition, the cloned cDNA should hybridize to mRNA whose size, tissue, and species distribution are not inconsistent with the structure and function of the protein. Finally, the properties of the polypeptide predicted by the nucleotide sequence of the cDNA should be in accord with those of the authentic protein. For example, the vast majority of secreted or plasma membrane proteins are derived from polypeptide precursors that carry a hydrophobic signal sequence at their amino termini. The absence of such a signal from the predicted polypeptide would therefore be cause for concern. Similarly, the predicted size of the polypeptide, its overall charge and amino acid composition, the number of potential glycosylation sites, and the presence of hydrophobic regions that could serve as transmembrane anchors should be consistent with the known properties of the authentic protein.

Protocols for cDNA Cloning

In the remainder of this chapter, we describe in detail the most effective methods to construct cDNA libraries in bacteriophage λ vectors. The scheme is based on the procedures of Gubler and Hoffman (1983) and Huynh et al. (1985) and has become the standard procedure used to synthesize and clone double-stranded cDNA. The basic steps in this scheme are:

1. Oligo(dT)-primed synthesis of the first strand of cDNA with reverse transcriptase
2. Synthesis of the second strand of cDNA (using RNAase H and *E. coli* DNA polymerase I), including the repair reaction with bacteriophage T4 polynucleotide kinase and *E. coli* DNA ligase
3. Methylation of *Eco*RI sites
4. Ligation to phosphorylated *Eco*RI linkers and digestion of linkers to yield cohesive termini
5. Size selection of cDNA
6. Ligation to bacteriophage λ arms, packaging of DNA into bacteriophage λ particles, and test plating on *E. coli*
7. Analysis of cDNA inserts (bacteriophage λ minipreps)
8. Generation of a complete cDNA library
9. Amplification of cDNA libraries

Virtually all of these steps can be monitored by control reactions. Furthermore, because these methods have been in common use for several years, most of the problems have been uncovered and solved. However, with the recent development of bacteriophage λ vectors with *Not*I or *Sal*I sites, it is often possible to simplify the basic scheme outlined above by using *Not*I or *Sal*I linkers instead of *Eco*RI linkers. Because the chance is small that the cDNA of interest will be cleaved by such enzymes, the methylation reaction with M.*Eco*RI methylase may be eliminated. The modifications that permit the use of *Not*I or *Sal*I linkers are marked at the appropriate points in the description of the basic protocol.

CONSTRUCTING cDNA LIBRARIES IN BACTERIOPHAGE λ VECTORS

Precautions

1. Making cDNA libraries is an expensive and time-consuming endeavor. To minimize the possibility of mistakes, many laboratories prepare ahead of time a set of reagents, buffers, and substrates that are used for no other purpose than cDNA cloning. This eliminates delays between one step in the protocol and the next and reduces the chance of accidental contamination by nucleases or exogenous nucleic acids. There are many anecdotes of cDNA libraries containing DNA sequences that had their origin in restriction enzymes and solutions that had been contaminated by fellow laboratory workers. cDNA cloning is particularly vulnerable to such contamination because it involves the manipulation of small amounts of cDNA through a succession of enzymatic reactions. The way that these reactions are arranged can lead to the efficient salvage of contaminating DNA. For convenience, a list of the critical materials, reagents, and buffers that are used to prepare and clone cDNA are presented in Table 8.1. Whenever possible, the solutions should be sterilized by autoclaving.
2. Carry out all operations in sterile microfuge tubes, using sterile pipettes and pipette tips.
3. In such a long series of enzymatic reactions and manipulations, there are many points at which potential problems may arise. However, by holding firmly to the rule of checking the products of each reaction, it is almost always possible to avoid ruination. Three commonly used routines for minimizing difficulties are:

   - *Checking the products of each reaction before proceeding to the next.* Although attractive in theory, this method of procedure slows down cDNA cloning to an unacceptable rate. However, there are several critical steps in the protocol at which we strongly recommend that checks (which are described at the appropriate points in the text) be carried out before proceeding further. These are:

Step	Check
Synthesis of the second strand of cDNA	Size and yield of product
Methylation of double-stranded DNA	Resistance of DNA to subsequent cleavage
Digestion of linkers with restriction enzymes	Cleavage of unmethylated test DNA
Ligation of cDNA to bacteriophage λ arms	Test ligation/packaging

   Small samples of every reaction should be saved for later analysis, if it should become necessary to pinpoint where problems may have occurred.

   - *Setting up a series of small-scale pilot reactions 1 or 2 days ahead of the time that these reactions will be used to synthesize the main batch of cDNA.* This procedure allows any potential problems to be identified and solved without putting the entire experiment at risk.

   - *Carrying out two sets of full-scale reactions, the second of which is begun 2 days after the first.* The second batch of cDNA acts as insurance

against unexpected loss of the first and guarantees that no more than 2 days will be lost.

4. Most losses of cDNA occur during precipitation with ethanol. Taking the following precautions helps to avoid this problem:

   - After adjusting the salt concentration of the DNA solution to 0.3 M sodium acetate or 2 M ammonium acetate and adding 2 volumes of ethanol, mix the contents of the tube very well. Stand the tightly closed tube at room temperature for at least 15 minutes, and then centrifuge at 12,000*g* for at least 15 minutes at 4°C. Under these conditions, even very small quantities of DNA (10 ng) are precipitated and there is no need to use a carrier.

     Some workers prefer to freeze the ethanolic mixture at −70°C (in a dry-ice/ethanol bath) for 15 minutes or to store it for more prolonged periods at −20°C before centrifugation. In our hands, these procedures do not improve the efficiency with which DNA is precipitated by ethanol.

   - Save the ethanol solution until you are sure that all of the DNA has been recovered. This is especially important after precipitates of DNA have been washed with 70% ethanol—treatment that often loosens the precipitates from the wall of the tube.

   - Make sure you redissolve all of the cDNA after ethanol precipitation. The precipitated DNA is not all found at the bottom of the tube—about 30–40% is plastered on the wall. This DNA is redissolved by rolling a bead of solvent several times over the appropriate segment of the wall. Use a pipette tip to roll the bead of solvent. Check that no detectable radioactivity remains in the tube after the redissolved cDNA has been removed.

TABLE 8.1 Reagents Used to Synthesize and Clone cDNA

Actinomycin D	Required only if self-priming of second-strand cDNA synthesis in unacceptably high (see note to step 5g on page 8.65). Preparations of actinomycin D supplied by pharmaceutical manufacturers for therapeutic uses often contain additional substances such as sugars and salts. Such preparations can be used to suppress self-priming as long as the concentration of actinomycin D is verified by measuring the absorbance of the stock solution at 440 nm. The molar extinction coefficient of pure actinomycin D (m.w. = 1255) in aqueous solution is 21,900. The absorbance at 440 nm of a solution containing 1 mg/ml of the drug is therefore 0.182. Stock solutions of actinomycin D are stored at −20°C in foil-wrapped tubes. **Caution:** Actinomycin D is a teratogen and carcinogen. Stock solutions should be prepared, wearing gloves and a mask, in a chemical hood, not on an open bench.
S-Adenosyl-L-methionine	Iodide salt, grade I. Make a stock solution (20 mM) in 5 mN H_2SO_4, 10% ethanol. Store in aliquots at −20°C. New England Biolabs provides a solution (30 mM) of *S*-adenosyl-L-methionine when you buy M.*Eco*RI methylase.
[α-^{32}P]dCTP	400 Ci/mmole, 10 μCi/μl in aqueous solution. [α-^{32}P]dATP and [α-^{32}P]dTTP should not be used as radioactive tracers to monitor synthesis of the first and second strands of cDNA, since they may be incorporated preferentially into the cDNA derived from the poly(A)$^+$ tract at the 3′ terminus of the mRNA.
ATP	Make 50 mM and 10 mM stock solutions as described for dNTPs. Store in aliquots at −70°C.
Bacteriophage λ arms	If you plan to make a large number of libraries, it is much cheaper to prepare arms by agarose gel electrophoresis (see Chapter 2) than to purchase them. For occasional users, dephosphorylated arms for some vectors are available from Stratagene. Whatever the source of arms, it is important to carry out a series of pilot reactions to check that they can be ligated to foreign DNA and packaged efficiently into infectious bacteriophage particles.
Bacteriophage T4 DNA ligase	Most manufacturers purify the enzyme from strains of *E. coli* that express the cloned gene at a high level. At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units. Bacteriophage T4 DNA ligase is supplied in concentrated solution (1–5 units/μl). This should be diluted and stored at a concentration of 100 units/ml in 20 mM Tris · Cl (pH 7.6), 60 mM KCl, 5 mM dithiothreitol, 500 μg/ml bovine serum albumin, 50% glycerol. Bacteriophage T4 DNA ligase is stable at this concentration in this buffer for 3 months at −20°C.
Bacteriophage T4 DNA polymerase	Most manufacturers purify the enzyme from *E. coli* infected with a mutant of bacteriophage T4 defective in gene *32* (Panet et al. 1973).
Bacteriophage T4 polynucleotide kinase	New England Biolabs sells enzyme isolated from a strain of *E. coli* lysogenic for a transducing bacteriophage λ that produces large amounts of bacteriophage T4 polynucleotide kinase.
β-Mercaptoethanol (0.3 M)	Make a fresh solution just before use by adding 300 μl of the 14.4 M solution supplied by the manufacturer to 14.1 ml of H_2O.

Chloroform

Deoxyribonucleoside triphosphates

Dissolve each dNTP in water at an approximate concentration of 100 mM. Using 0.05 M Tris base and a micropipette, adjust the pH of each of the solutions to 7.0 (use pH paper to check the pH). Dilute an aliquot of the neutralized dNTP appropriately, and read the optical density at the wavelengths given in the table below. Calculate the actual concentration of each dNTP. Dilute the solutions with water to a final concentration of 50 mM dNTP. Store each separately at −70°C in small aliquots.

Base	Wavelength (nm)	Extinction coefficient (ϵ) (M^{-1} cm^{-1})
A	259	1.54×10^4
G	253	1.37×10^4
C	271	9.10×10^3
T	260	7.40×10^3

For a cuvette with a path length of 1 cm, absorbance = ϵM.

100 mM stock solutions of each dNTP are commercially available (Pharmacia) if you do not want to prepare your own. Use the four stock solutions to make a single working solution containing each of the four dNTPs at a concentration of 5 mM. Store the mixture in aliquots at −70°C.

Dithiothreitol (0.1 M)

Make a stock solution in water. Store in small aliquots at −20°C.

E. coli

Use strains C600 (BNN93) for growth and BNN102 (C600*hflA*) for screening of cDNA libraries constructed in bacteriophage λgt10. Use strain Y1090*hsdR* for growth and screening of cDNA libraries constructed in bacteriophage λgt11. Use strain BB4 for growth and screening of cDNA libraries constructed in λZAP and λZAPII or strain XL1-Blue for λZAPII. (Strain XL1-Blue supports vigorous growth of λZAPII but not λZAP.)

E. coli DNA ligase

This enzyme is purified from *E. coli* by several manufacturers.

E. coli DNA polymerase I

Several manufacturers sell enzyme isolated from a strain of *E. coli* lysogenic for a bacteriophage λ that carries the gene (*polA*) that codes for the holoenzyme of *E. coli* DNA polymerase I (Murray and Kelley 1979). Most commercial suppliers sell this enzyme in buffer containing 50% glycerol. Usually, 1 μl of the preparation contains 5 units of enzyme (for a definition of a unit, see Richardson et al. 1964).

*Eco*RI

New England Biolabs sells enzyme isolated from a strain of *E. coli* that expresses the *RI* restriction and modification genes from a multicopy plasmid.

M.*Eco*RI methylase

New England Biolabs sells enzyme isolated from a strain of *E. coli* that expresses the methylase gene from a multicopy plasmid.

EDTA (0.5 M; pH 8.0)

IPTG

Isopropylthio-β-D-galactoside (m.w. = 238.3). Make a solution of IPTG by dissolving 2 g of IPTG in 8 ml of H_2O. Adjust the volume of the solution to 10 ml with H_2O and sterilize by filtration through a 0.22-micron disposable filter. Dispense the solution into 1-ml aliquots and store them at −20°C.

KCl (1 M)

Linearized plasmid DNA

This is used to check the efficiency of methylation of *Eco*RI sites (see page 8.66–8.67) and to check the efficiency of digestion following linker addition (see page 8.69). The plasmid should therefore contain at least one *Eco*RI site located some distance from the ends of the linear DNA (e.g., Xf3 or pBR322 linearized by digestion with *Pst*I). Digest 1 μg of the plasmid DNA to completion with *Pst*I. Purify the DNA by extraction with phenol:chloroform and precipitation with ethanol. Redissolve the DNA in 5 μl of TE (pH 7.6). Store at −20°C.

$MgCl_2$ (1 M)

Also prepare working solutions of 10, 100, and 250 mM $MgCl_2$.

TABLE 8.1 *(continued)*

NaCl (5 M)	Make a 50 mM stock solution of nicotinamide adenine dinucleotide in water. Store in aliquots at −70°C.
NAD	
$(NH_4)_2SO_4$ (1 M)	These are available from several manufacturers of restriction enzymes.
*Not*I and *Sal*I	This is sold by Pharmacia. Prepare a stock solution (1 mg/ml in 10 mM Tris · Cl [pH 7.4]). Store in aliquots at −70°C.
Oligo$(dT)_{12-18}$	Packaging extracts are much cheaper to make than to buy. If you plan to construct more than one or two cDNA libraries, it would be worthwhile making a large batch of packaging extract (see Chapter 2, pages 2.98–2.107). However, Gigapack Gold (sold by Stratagene) has a higher efficiency of packaging than most extracts made by occasional users and is recommended for those who make libraries only occasionally.
Packaging extracts for bacteriophage λ	
Phenol:chloroform	Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris · Cl (pH 7.6). Store the equilibrated mixture under an equal volume of 0.01 M Tris · Cl (pH 7.6) at 4°C in dark glass bottles.
Poly$(A)^+$ RNA	Approximately 10 μg of poly$(A)^+$ RNA is required to synthesize enough double-stranded cDNA to construct a large library. (One confluent 90-mm plate of cultured mammalian cells yields 1–2 μg of poly$(A)^+$ RNA.) The synthetic reactions will work if less template is available, but losses at each stage will be proportionately higher. Before proceeding, the integrity of the poly$(A)^+$ RNA should be checked by (1) agarose gel electrophoresis (see Chapter 7, page 7.31), (2) translation in a cell-free system followed by SDS-polyacrylamide gel electrophoresis of the resulting polypeptides (see page 8.4), and (3) analysis of the size of the first strand of cDNA synthesized in a pilot reaction (see pages 8.61–8.62).
Reverse transcriptase	In our hands, reverse transcriptase prepared by expression of a gene cloned from a murine retrovirus is better than the enzyme purified from particles of an avian retrovirus. The two enzymes display different pH, salt, and temperature optima, and the reaction conditions given in the experimental protocol on page 8.60 should be used with the murine enzyme only. If the avian enzyme is to be used, prepare the reaction buffer as described on page 8.62. Murine reverse transcriptase expressed from cloned genes is available from a number of manufacturers. Two different clones are currently used to produce the enzyme commercially. The clone used by Pharmacia and Stratagene produces a fusion protein that contains nine amino acids encoded by pBR322 sequences. The other clone was constructed by BRL. The differences between these clones may account for the disparity in units of reverse transcriptase recommended for first-strand synthesis (BRL: 200 units/1–5 μg RNA; Pharmacia: 20–30 units/μg; Stratagene: 200 units/1–5 μg).
RNAase H	This enzyme is purified from *E. coli* by several manufacturers.
RNAase inhibitor	This protein of ~51 kD, purified from human placenta, is a noncompetitive inhibitor of pancreatic RNAase. This inhibitor should be used at a final concentration of 0.5 units/μl of reaction mixture and should be added to the mixture before reverse transcriptase. The inhibitor requires sulfhydryl reagents (1 mM dithiothreitol) for activity and, in fact, denatures irreversibly in the absence of these agents. The inhibitor should be stored at −20°C in glycerol. Do not use preparations of inhibitor that have been frozen and thawed many times; this denatures the protein and releases any bound RNAase that may contaminate the preparation. The protein does not inhibit the activity of RNAase H. It is sold by several manufacturers.

Sephadex G-50	Add Sephadex G-50 (medium) to distilled sterile water (10 g of dry powder yields 160 ml of slurry). Wash the swollen resin with distilled sterile water several times to remove soluble dextran, which can create problems by precipitating during ethanol precipitation. Finally, equilibrate the resin in TE (pH 7.6), autoclave (10 lb/sq. in. for 15 minutes), and store at room temperature.
Sepharose CL-4B	Wash the paste supplied by the manufacturer several times in sterile TE (pH 7.6) containing 0.1 M NaCl. This washing removes inhibitors of ligation present in some batches of the gel.
Sodium acetate (3 M; pH 5.2)	
Synthetic linkers	Phosphorylated linkers are sold by several manufacturers. If possible, obtain linkers that are at least 4–8 nucleotides longer than the site recognized by the restriction enzyme. Many restriction enzymes inefficiently cleave recognition sites located very close to the ends of DNA molecules.
TE (pH 7.6 and pH 8.0)	0.01 M Tris·Cl (pH 7.6 or pH 8.0), 0.001 M EDTA (pH 8.0).
Trichloroacetic acid (TCA)(10%)	
Tris base (2 M)	
Tris·Cl (2 M; pH 7.4)	
Tris·Cl (1 M; pH 7.6)	
Tris·Cl (2 M; pH 8.0)	
Tris·Cl (1 M; pH 8.3)	This reagent is used to buffer the synthesis of the first strand of cDNA catalyzed by reverse transcriptase purified from avian retroviruses. Since the enzymatic reaction is carried out at 42°C and the pH of Tris solutions is particularly temperature-dependent, the pH of the stock buffer should be adjusted at this temperature. A different buffer is used for murine reverse transcriptase. BRL supplies a 5× reaction buffer for synthesis of the first strand of cDNA when you order murine enzyme.
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside. Make a stock solution by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution. Use a glass or polypropylene tube. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and should be stored at −20°C. It is not necessary to sterilize X-gal solutions by filtration.